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PHYSIOLOGY OF THE REPRODUCTIVE CYCLE IN THE COCKROACH *BYRSOTRIA FUMIGATA* (GUÉRIN)

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Previous studies on reproduction in cockroaches and in certain other insects have implicated juvenile hormone in the control of various processes occurring during the female reproductive cycle: yolk formation (reviews: Wigglesworth, 1964; Engelmann, 1968), vitellogenic blood protein secretion (Coles, 1964; Minks, 1967; Bell, 1969a; Engelmann, 1969; Scheurer, 1969a), colleterial gland activity (Willis and Brunet, 1966; Bodenstein and Shaaya, 1968; Shaaya and Bodenstein, 1969) and sex pheromone secretion (Barth, 1962, 1968; Emmerich and Barth, 1968). These processes have not been investigated, however, with regard to their integration in the reproductive cycle. It is the purpose of this communication to describe the reproductive cycle of *Byrsotria fumigata,* an ovoviviparous cockroach, and to report observations on the factors which control the initiation and termination of these four processes during the cycle.

The cycle of *B. fumigata* differs from that of the more primitive oviparous forms in that the oothecae are oviposited into a brood sac and incubated within the female until hatching rather than being produced at frequent intervals and oviposited externally (Fig. 1). Oocyte development and the other reproductive processes characteristic of the preoviposition period are inhibited during the lengthy term of pregnancy; after parturition the various reproductive processes recommence. Owing to the greater complexity of their cycle, the ovoviviparous cockroaches are more interesting than oviparous species from the viewpoint of the physiological control mechanisms operating in the regulation of the cycle. Female reproductive cycles in cockroaches have been discussed in relation to sexual behavior (Barth, 1968) and also with regard to the evolution of the cycle (Roth, 1970). The oviparous type of cycle, as exemplified by *Periplaneta americana,* has been treated by Bell (1969b) with special reference to oocyte development.

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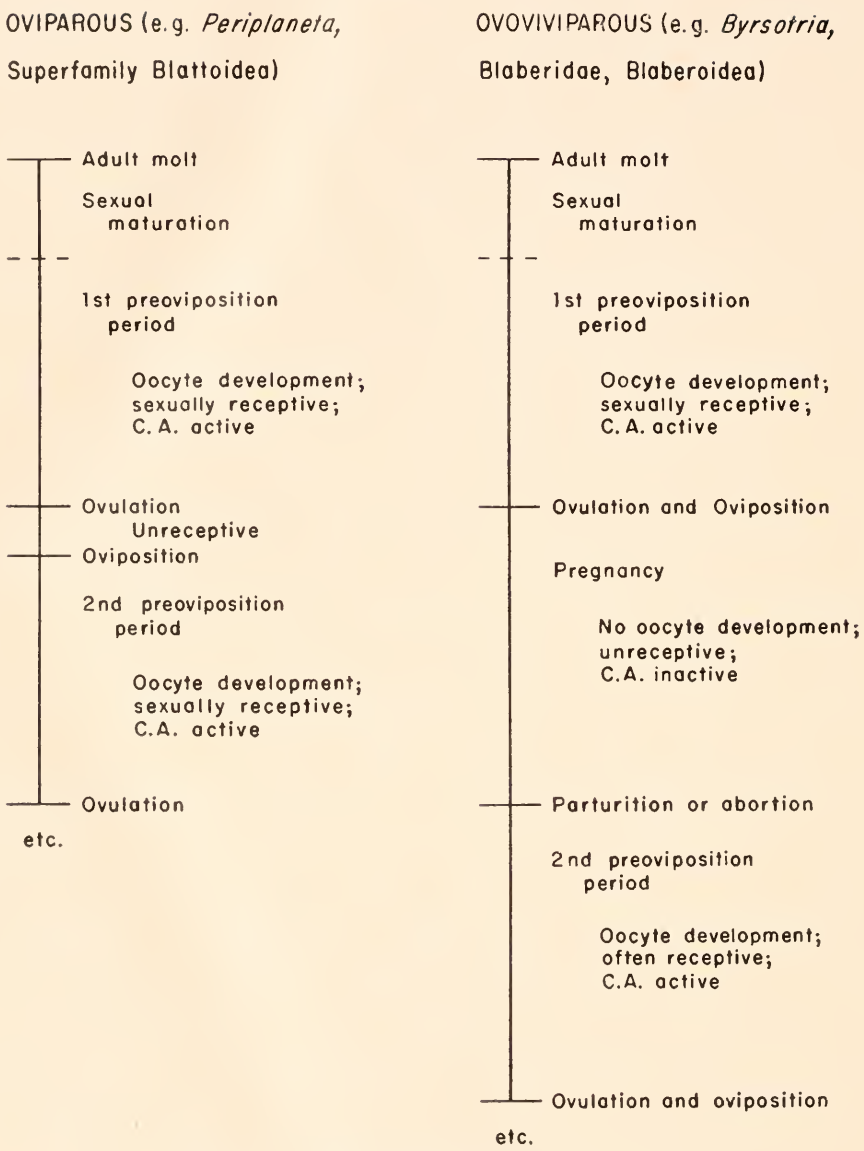

FIGURE 1. Reproductive cycles of cockroaches.

METHODS AND MATERIALS

1. Insect material

B. fumigata nymphs were maintained in plastic cages with access to food and water as described previously by Barth (1964). All females used in the experiments were removed from the cages within 24 hours after they emerged as adults

(designated day 0) and were thereafter housed individually in 250 ml beakers. The females were mated between days 12 and 16 after emergence and spermatophore insertion was used as a criterion for successful mating. Experimental females were bled and dissected within 6 hours following the assay for pheromone, and the blood was stored at −5° C. The clotted cells were separated from the serum by allowing the serum to seep out of the clot in a horizontal test tube. Oocytes and colleterial glands were excised from dissected females and extracted as described below.

2. Pheromone assay

The sex pheromone assay, previously described by Barth (1961, 1962), consisted of removing a filter paper from a beaker containing a female and placing it in or suspending it a few inches above a container of males for two minutes. If the female was secreting pheromone (absorbed by the filter paper) the paper elicited courting responses in groups of adult males. In the present study an additional assay for pheromone secretion was used; the virgin female herself was placed in or held a few inches above a container of males. In this way the presence of the pheromone on the cuticle of the female could be assayed by the male response.

3. Yolk deposition

The criteria used to measure the initiation and progress of yolk deposition were the appearance of refractile yolk spheres in the oocyte cortex, appearance of significant amounts of extractable protein in the oocytes and changes in oocyte volume (Bell, 1969b).

4. Vitellogenin secretion

The Oudin (1948, 1952) technique was used to resolve the resulting antigen-antibody reactions when blood or yolk fluid was reacted with an antiserum containing antibodies homologous to the yolk antigens. The antiserum was derived from the blood of rabbits which had been injected with the proteinaceous portion of *B. fumigata* yolk fluid. The yolk extracts and the antiserum were prepared

TABLE I

Concentration of yolk antigens in cockroach blood relative to the concentration in a yolk fluid standard

	Antigen (% concentration)			
	A	B	C	D
Yolk fluid	100.00	100.00	100.00	100.00
Ovariectomized female (day 16)	4.00	10.90	86.40	100.00
Adult female (day 15)	1.20	4.30	101.05	118.00
Pregnant female	0.04	0.03	93.70	87.00
Adult male (day 15)	0.00	0.00	101.05	117.00
Female nymph	0.00	0.00	94.00	94.00

using techniques previously described for the preparation of an antiserum against yolk proteins of *Periplaneta americana* (Bell, 1970).

Glass tubes (6 × 0.5 cm) were half-filled with diluted antiserum mixed with agar (1:10). When the antiserum-agar mixture was solidified, yolk fluid or cockroach blood was layered on the tubes and a topping of mineral oil was added to prevent evaporation. As the antigens diffused into the antiserum-agar, four precipitin bands with sharp leadings edges were observed. Of the four bands, 2 were of light density (antigens C and D) and resulted from precipitin tests with yolk fluid or blood from male, female or immature cockroaches. Table I shows that the

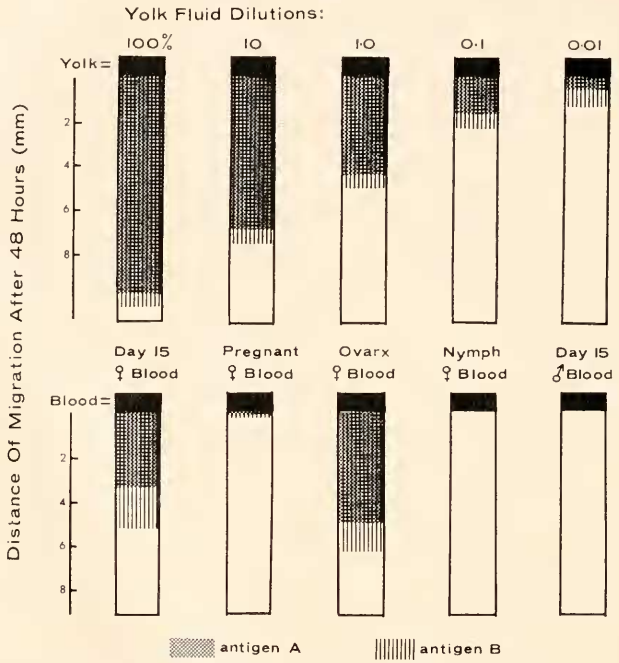

FIGURE 2. Distance of migration by precipitin bands in Oudin tubes containing antiserum absorbed with male blood and mixed with agar.

concentration of antigens C and D is similar in yolk fluid and blood from males, females and nymphs. Added to the fact that antigens C and D do not accumulate in ovariectomized female blood and are not selectively sequestered by the oocytes (Table I), these data suggest that antigens C and D are not female-specific vitellogenic blood proteins, but occur as minor components of the yolk.

Antigens A and B which produce the two dense precipitin bands, on the other hand, are taken up by the oocytes over a considerable concentration barrier and accumulate in the blood of ovariectomized females. Moreover, these antigens are female-specific, occur only in adult, vitellogenic females and, as indicated by their dense bands of precipitation in Oudin tubes, comprise a major portion of the yolk protein complement. In view of these findings, the antigens responsible for the

formation of precipitin bands A and B are referred to here as vitellogenins. This term was applied by Pan, Bell and Telfer (1969) to designate a functionally discrete group of insect blood proteins which are female-specific and which are selectively sequestered by the oocytes.

Figure 2 illustrates the results of using the Oudin test to measure the concentration of vitellogenins A and B in *B. fumigata* blood or serially diluted yolk fluid (in this case the antiserum was previously absorbed with male blood in order to remove antibodies homologous to antigens C and D). It has been shown by Oudin that a linear relationship exists between the logarithm of the antigen concentration and the value K (distance of migration [mm] by the leading edge of a precipitin band divided by the square root of time [min]). With regard to the above relationship, the distance of migration of the precipitin bands in tubes containing serial dilutions of yolk fluid were measured and these distances were divided by the square root of time; the resulting K values were plotted against the concentration

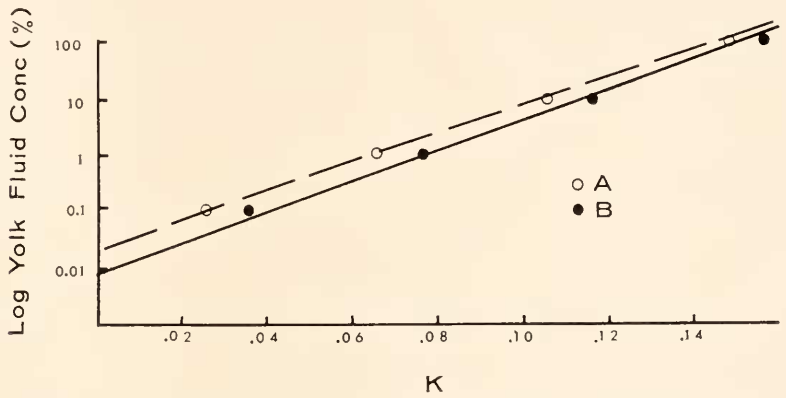

FIGURE 3. Logarithm of yolk fluid concentration plotted against K values [distance of migration of precipitin bands (in mm) divided by the square root of the time interval (in minutes)].

of yolk fluid (Fig. 3). The rates of migration of the bands in tubes layered with blood from 15-day females, pregnant females and 16-day ovariectomized females were also measured and the concentration of the vitellogenins in these solutions, relative to their concentrations in the yolk fluid, was ascertained by referring to the standard curve shown in Figure 3. Table I shows the concentration of the vitellogenins in the solutions tested.

In the studies presented in this paper, the relative concentrations of the vitellogenins were measured in the blood of females during the reproductive cycle in order to ascertain the relationship between the yolk precursor secretion cycle and other reproductive processes.

5. Colleterial gland glucoside activity

The spectrophotometric glucoside assay of Willis and Brunet (1966) was employed to measure the secretory activity of colleterial glands. The left colleterial

glands were excised and homogenized in 2 ml of saturated ammonium sulfate. 0.2 ml of the supernatant was mixed with 2.8 ml of distilled water and the solutions were read against appropriate blanks in a Carey recording spectrophotometer.

Colleterial gland extracts contained the glucoside (G1-A) which was previously characterized by paper chromatography (Stay and Roth, 1962) and was found to have an absorption spectrum at 2770 Å. A second component, with a peak of 2850 Å, was observed in glands excised from newly emerged females (Days 0 to 8). Glucoside units (a change in O.D. of 0.001) were measured in colleterial glands from females during the reproductive cycle.

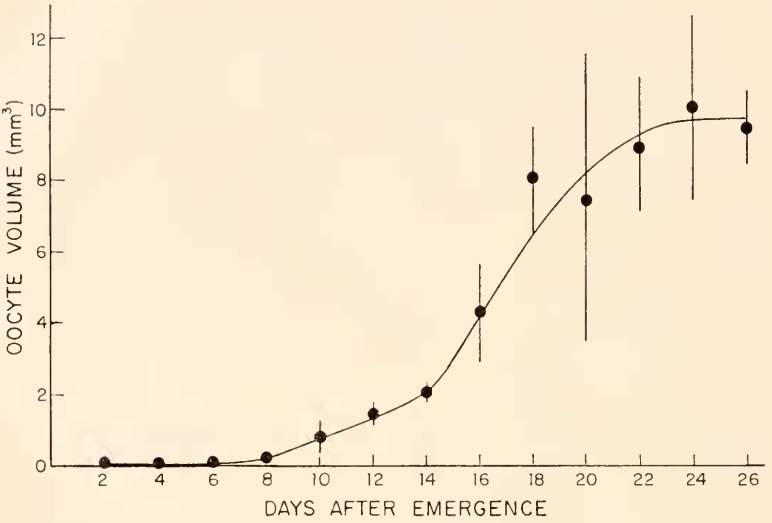

FIGURE 4. Changes in oocyte volume during the first preoviposition period. Vertical lines are standard deviations. Each point represents the average of 10 to 20 females.

The width of the left colleterial gland tubules was also used as an indication of gland activity. In newly emerged females the gland tubules are white and have an average width of 0.1 mm, while in vitellogenic females the tubules are bluish green and have an average width of 0.6 mm.

RESULTS

1. Yolk deposition

The onset of yolk deposition occurs on about day 5 after ecdysis; the basal oocytes are characterized by detectable quantities of extractable vitellogenin, an increase in volume and the appearance of refractile yolk spheres in the oocyte cortex. Rapid deposition of vitellogenic blood proteins occurs during days 14 to 22, followed by a period of less intense deposition and terminating completely by day 26. Oocytes with an average volume of 10 mm^3 were ovulated between days 23 and 30 (average of 26) after emergence (Fig. 4). Employing essentially similar culture conditions and techniques as in the present study, Roth and Stay (1962)

reported that in their colonies the mated females oviposited between days 26 and 41 (average of 32.4). This apparent reduction in the period required for oocyte maturation in insects cultured over a long period of time has also been observed in *Schistocerca gregaria* (L. Hill, University of Sheffield, personal communication).

Female *B. fumigata* are normally receptive to courting males between days 10 and 30 after emergence. In females which fail to mate, the oocytes often fail to develop or may be resorbed after partial development; moreover, nearly all virgins (including those which do oviposit) lack the synchronous oocyte growth observed in mated females. Virgin females which oviposit do so slightly later than mated females (Roth and Stay, 1962).

2. Vitellogenin secretion

The blood of females on day 4 or 5 after emergence contains slight, but unmeasurable quantities of vitellogenin (less than 0.001% relative to the yolk fluid standard), suggesting that these yolk precursors are present, but only in trace amounts. Significant levels of both vitellogenins appear in the blood of most females on day 7 or 8. Antigen B is detected about 24 hours prior to the appearance of antigen A, and throughout the cycle antigen B occurs in higher concentra-

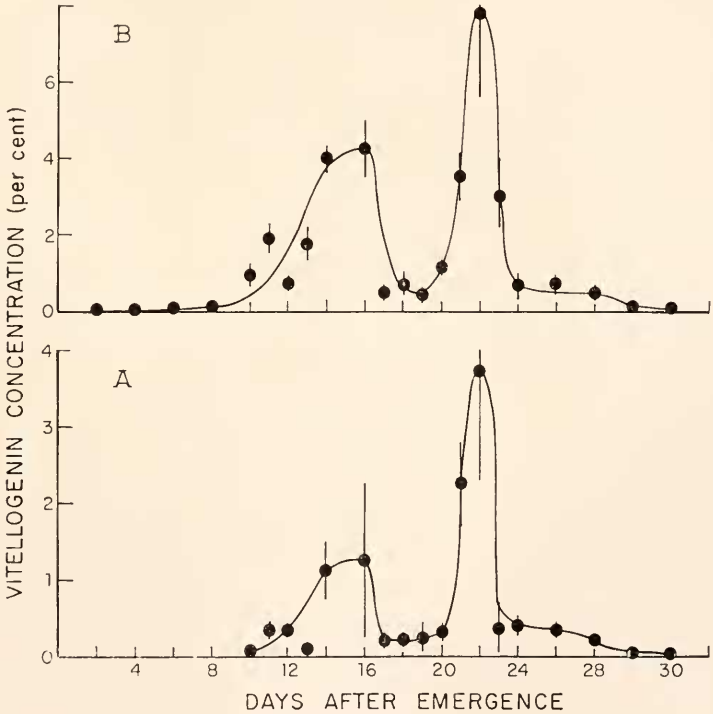

FIGURE 5. Changes in blood vitellogenin concentration during the first preoviposition period. Vertical lines are standard deviations. Each point represents an average of 10 to 15 females.

tion than antigen A (relative to their concentration in the yolk fluid standard). From the time of their first appearance in female blood the vitellogenins increase synchronously to a peak on day 16 (Fig. 5), drop to a low level by day 18, increase to a second peak at day 22 and then decrease in concentration until ovulation. Finally, in most females the vitellogenins disappear entirely from the blood during pregnancy; indeed, by the tenth day of pregnancy the vitellogenins could not be detected in 80% of the females tested.

The trough in the vitellogenin curve between days 17 and 20 corresponds to the period of most intense yolk investment. During this period the oocytes increase in volume by 4.00 mm^3, an increment which is greater than that of the previous or the succeeding 4 day period. Secondly from days 20 to 24 the rate of increase in oocyte volume is substantially less than during any other period in the vitellogenic cycle; this hiatus in yolk deposition may account for the observed replenishment of blood vitellogenin which reaches a peak on about day 22. Resorption of some basal oocytes which commonly occurs even in mated females towards the end of the vitellogenic period, may also contribute to the second peak in vitellogenin concentration.

Measurements were made of the vitellogenin concentration in the blood of ovariectomized females. The accumulation of the protein yolk precursors in castrated females (which reaches a concentration of 4.0% [A], and 10.9% [B] at day 16) gives credence to the role of these proteins in yolk deposition; moreover, the observation that the vitellogenin concentration of ovariectomized females declines to 1.4% [A], and 4.6% [B] at the time when normal females are initiating egg incubation suggests that the observed decreasing concentrations of blood vitellogenin towards the end of vitellogenesis is due to processes in addition to the removal of these proteins from the blood by yolk-forming oocytes.

3. Pheromone production

On the basis of the filter paper assay it was reported that 90% of normal virgin females initiated pheromone production between 10 and 30 days after the imaginal molt (Barth, 1961, 1962). However, it was also noted that females maintained with males from emergence occasionally mated as early as day 4 (Barth, 1962). This observation together with other studies demonstrating the importance of the sex pheromone for the release of male courtship behavior (Barth, 1964) led to the conclusion that the pheromone is very likely present on the body surface of the female at an earlier age than that at which it is detectable on filter paper. For this reason the pheromone assay employing the female as described above was extensively employed in these studies. When introduced into a chamber of males, the test females usually showed the immobilization reaction (flattening against the substratum and withdrawing the antennae laterally beneath the pronotal shield—Barth, 1964), or less commonly attempted to escape by hiding under a nearby object. Such females were invariably sexually unreceptive and behaviorally were essentially as inert as filter papers, hence any stimuli presented to the males are highly likely to have been chemical alone.

Table II indicates that by day 4 a sufficient quantity of pheromone was present on the cuticle of 5% of the females tested to elicit a courting response by adult males. On day 10, 94% of the females placed in male cages elicited the male court-

TABLE II

*Initiation of pheromone production**

	Days after emergence											
	1	2	3	4	5	6	7	8	9	10	11	12
% of females with detectable cuticular pheromone	0	0	0	5	17	32	52	74	84	94	100	100

* Represents data from 65 females.

ing response. It is clear that by means of this assay procedure, data can be obtained which bring the initiation of pheromone secretion into line with the initiation of other reproductive processes at the onset of the reproductive cycle. Barth (1970) reported that the pheromone is a product of some portion of the female genital tract other than the colleterial glands; thus according to our current hypothesis, the pheromone is secreted through the genital opening and is selectively adsorbed onto the surface of the cuticle. It is also adsorbed onto the surface of other objects in the vicinity (*i.e.*, filter papers). Whether such objects possess sufficient pheromone activity to stimulate the males depends upon the rate of pheromone output by the female relative to the evaporation and/or breakdown rate of the pheromone. Output rates seem to vary considerably and may be characteristic of individual females (Barth, 1962).

As reported by Barth (1968) and as shown in Table III, pheromone production ceases within a few days after mating. In some individuals it may cease as early as the first day and in no case has it been observed to continue beyond the third day following mating. By contrast virgin females continue to produce the pheromone until they ovulate; moreover, if they fail to ovulate on schedule (Roth and Stay, 1962), they may continue to produce sex pheromone for several weeks.

A related event of importance which occurs early in the reproductive cycle of the female is the onset of sexual receptivity. Previous work on sexual receptivity suggests that in contrast to the other four processes under discussion, it is not regu-

TABLE III

*Cessation of pheromone production by females after mating**

Number of females tested	Age (in days after mating) when first tested	% females with positive pheromone
66	0†	100
4	1	0
31	2	0
16	3	33‡
15	4	0

* Filter paper assay used in all tests. Females mated at 10–16 days of age one day after demonstrating pheromone production by means of the filter paper assay.

† Indicates the day on which females were mated.

‡ These females all ceased pheromone production on day 4 after mating.

lated directly by the corpora allata (Roth and Barth, 1964; Barth, 1968; Engelmann and Barth, 1968). The results of the female pheromone assay above indicate that the female herself is not receptive at the time when sex pheromone on the surface of her cuticle makes her attractive to males. In view of our interest in the sequence of reproductive events we continued to test a small number of active females daily for signs of receptivity. All six animals so tested showed antennation and mounting of displaying males within the two minute test period on the second day following appearance of pheromone on the cuticle. Thus females appear to reach a high level of sexual receptivity about 2 days after the onset of pheromone secretion. It may be noted that this is likely to be several days before the average onset date for pheromone secretion as determined by the filter paper assay. Herein lies the probable explanation for the accounts in the literature of mating in *B. fumigata* females prior to the onset of pheromone production (Barth, 1962; Roth and Stay, 1962).

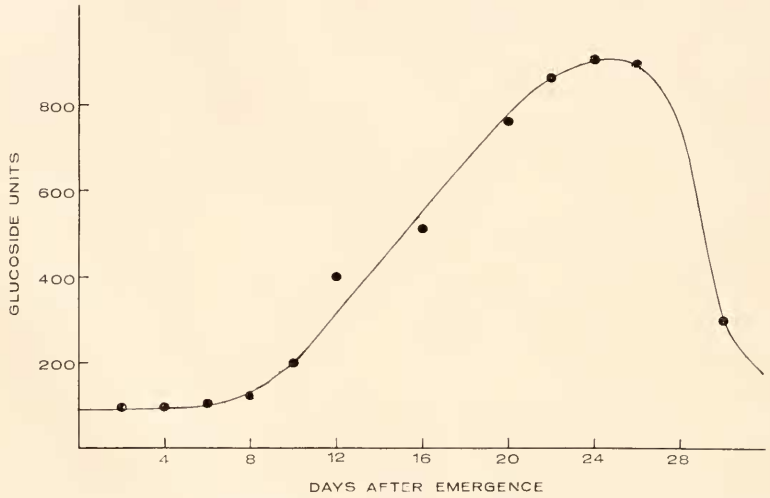

FIGURE 6. Changes in left colleterial gland glucoside concentration during the first preoviposition period. Each point represents the average glucoside units measured for the pooled glands of 5 females.

4. Colleterial gland activity

The left colleterial gland of *B. fumigata* secretes the structural protein of which the ootheca is composed and a glucoside of the phenolic tanning agent (Stay and Roth, 1962). Synthesis and storage of the protein is indicated by a bluish green color and increase in width of the gland tubules. The appearance of color and increase in colleterial gland size was observed to occur between days 4 and 6. In addition to the glucoside with an absorption peak of 2770 Å, the glands contained an unknown substance with an absorption peak of 2850 Å; this unknown was present in the left colleterial gland extracts (in saturated ammonium sulfate) until between days 6 and 8 at which time the unknown was replaced by the glucoside.

The presence of the left colleterial gland glucoside of *B. fumigata* (G1-A), identified chromatographically by Stay and Roth (1962), was first detected between days 6 and 8 after adult ecdysis. The units of activity increased linearly (Fig. 6) in mated females until a leveling off point occurred on about day 24. In virgin females the individual variation was very large owing to the low concentration of glucoside observed in many of these animals after day 15.

Following ovulation and oviposition there was a marked decline in glucoside activity, but some glucoside is apparently retained by the colleterial glands throughout the pregnancy period. It is interesting to note that the average period of pregnancy (53.1 days) observed in these studies is substantially shorter than the average pregnancy period of 76.2 days recorded by Roth and Stay (1962); these findings provide further evidence for the acceleration of reproductive processes in insects cultured for a number of generations.

Discussion

Four reproductive processes in the cockroach *B. fumigata* are known to be influenced by juvenile hormone (Bell and Barth, 1970). These processes appear to be initiated simultaneously in females on about the fifth day after adult emergence. An attempt was made in the present study to determine whether the initiation of any one process consistently preceded any other, but this was shown not to be the case.

A number of analogies between the reproductive cycle of *B. fumigata* and those of other cockroach species are revealed by the study reported here. The crucial difference between the two major cockroach groups is that the cycle of the family Blaberidae includes a period of pregnancy which is absent from the cycle of the superfamily Blattoidea and the family Blattellidae of the superfamily Blaberoidea (classification according to McKittrick, 1964) (see Fig. 1). This difference brings about obvious changes in the schedule of yolk formation, vitellogenin secretion, colleterial gland function and pheromone secretion; in *P. americana,* for example, the cycle of vitellogenin secretion and sequestration by the oocytes is continuous owing to the absence of a pregnancy period (Bell, 1969b). Among several consequences of a continuous cycle as exemplified by *P. americana* is the elimination of a peak in blood vitellogenin found in many *B. fumigata* females at the termination of yolk formation. In *P. americana* the second (penultimate) set of oocytes, which begins development prior to the termination of development in the first (basal) set, accumulates any vitellogenins which are not sequestered by the basal oocytes. On the other hand in *B. fumigata* there is a small quantity of blood vitellogenin which is not utilized and which remains in the blood during the first 10 to 20 days of pregnancy.

Another consequence of having continuous reproductive cycles and probably continuous juvenile hormone secretion, is that vitellogenin secretion and yolk deposition do not occur periodically, but seem to be maintained constantly throughout the reproductive life of the female (Bell, 1969b). Colleterial gland activity, on the other hand, proceeds somewhat differently for reasons of cyclic demand; during ootheca formation in *P. americana* the colleterial gland glucoside activity is reduced by 75%, but the supply is replenished within 2 days after ootheca formation (Willis and Brunet, 1966).

In *B. fumigata* the onset of pregnancy brings about a concomitant termination of vitellogenin secretion and yolk deposition; the same is true in *Leucophaea maderae,* another oviviparous species (Engelmann and Penney, 1966). The colleterial gland glucoside activity is reduced by 78% during ootheca formation in *B. fumigata,* but it is not until parturition that the gland begins to fill again; parturition is also the point in the reproductive cycle at which yolk deposition and vitellogenin secretion are again initiated.

Pheromone activity is first associated with the cuticle of day 4 females and may ultimately be detected on filter papers in beakers housing day 10 or older females. It is not known whether pheromone transfer to the filter papers is due to an increased pheromone output by the females or to a change in the structure of the pheromone molecule. The absence of a precise correlation between the pheromone secretion schedule and the schedule of female receptivity, as observed in our study, substantiates earlier work on *B. fumigata, Nauphoeta cinerea* and *L. maderae* (Roth and Barth, 1964; Barth, 1968), suggestive of different control mechanisms for the two processes.

The properties and functions of the two *B. fumigata* vitellogenic blood proteins suggest that they are analogous to similar proteins observed in *L. maderae* (Dejmal and Brookes, 1968; Engelmann and Penney, 1966; Engelmann, 1969), and *P. americana* (Bell, 1969b, 1970). In all three cases the vitellogenins require a high ionic strength for solubility and are selectively incorporated from the blood by yolk-forming oocytes. In the three species mentioned above, the two primary yolk components can be extracted only if the medium contains a salt concentration of at least 0.4 *M* NaCl. Whereas the vitellogenins are the primary yolk constituents, other protein components do exist in cockroach yolk as demonstrated electrophoretically or immunochemically by Adiyodi and Nayar (1967), Bell (1970), Nielsen and Mills (1968) and Scheurer (1969b). These proteins, however, are present in relatively small amounts and do not exceed their relative concentration in blood; hence they are not selectively sequestered by the oocytes.

With regard to the hormonal effect, it should be noted that even though the onset of juvenile hormone secretion is thought to occur at about the same time as the initiation of the various reproductive processes, it is impossible to determine at this time whether the hormone acts directly upon the ovaries (yolk deposition), fat body (yolk precursor synthesis), colleterial glands and pheromone gland; indeed, until the molecular effects of the hormone are further elucidated we will not know the precise relationship between the effector sites of juvenile hormone and the processes which we have studied. Specific effects of the hormone will be the topic of subsequent papers in this series.

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Summary

1. Four reproductive processes (vitellogenin secretion, yolk deposition, colleterial gland activity and pheromone production) are initiated on about the fifth day

after adult ecdysis. There is no apparent sequence of initiation of these processes except that vitellogenin secretion either precedes or occurs concomitantly with the initiation of yolk deposition.

2. Two blood proteins, which are female-specific yolk precursors (vitellogenins A and B), reach a concentration peak on day 16 and then decrease in response to rapid yolk deposition. A second peak on day 22 is thought to represent yolk precursors which were released to the blood as a result of the resorption of some basal oocytes. The vitellogenins are either absent or occur in trace quantities in the blood of pregnant females.

3. Sex pheromone is detectable on the surface of the cuticle by day 10 in 94% of the females tested. Following mating pheromone production is drastically curtailed.

4. Glucoside activity in colleterial glands increases almost linearly from day 8 to 24. After ootheca formation, which involves the utilization of colleterial gland secretion, there is a 78% reduction in glucoside activity.

5. The sequence of events in the reproductive cycle of females of *B. fumigata* is compared with that of other cockroaches.

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